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Research Note—

Protection Against Infectious Bursal Disease Virulent Challenge Conferred by a Recombinant Avian Adeno-Associated Virus Vaccine

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SUMMARY. The development and use of recombinant vaccine vectors for the expression of poultry pathogens proteins is an active research field. The adeno-associated virus (AAV) is a replication-defective virus member of the family *Parvoviridae* that has been successfully used for gene delivery in humans and other species. In this experiment, an avian adeno-associated virus (AAAV) expressing the infectious bursal disease virus (IBDV) VP2 protein (rAAAV-VP2) was evaluated for protection against IBDV-virulent challenge. Specific pathogen free (SPF) birds were inoculated with rAAAV-VP2 or with a commercial intermediate IBDV vaccine and then challenged with the Edgar strain. IBDV-specific antibody levels were observed in all vaccinated groups; titers were higher for the commercial vaccine group. The live, commercial vaccine induced adequate protection against morbidity and mortality; nevertheless, initial lymphoid depletion and follicular atrophy related to active viral replication was observed as early as day 14 and persisted up to day 28, when birds were challenged. No bursal tissue damage due to rAAAV-VP2 vaccination was observed. Eight-out-of-ten rAAAV-VP2-vaccinated birds survived the challenge and showed no clinical signs. The bursa:body weight ratio and bursa lesion scores in the rAAAV-VP2 group indicated protection against challenge. Therefore, transgenic expression of the VP2 protein after rAAAV-VP2 vaccination induced protective immunity against IBDV challenge in 80% of the birds, without compromising the bursa of Fabricius. The use of rAAAV virions for gene delivery represents a novel approach to poultry vaccination.

RESUMEN. *Nota de Investigación*—Protección contra un desafío virulento del virus de la enfermedad infecciosa de la bolsa conferida por una vacuna recombinante del virus adeno-asociado aviar.

El desarrollo y uso de vectores vacunales recombinantes para la expresión de proteínas de patógenos aviares es un área activa de investigación. El virus adeno-asociado aviar es un virus de replicación defectiva, miembro de la familia *Parvoviridae* que ha sido utilizado exitosamente para la transferencia de genes en humanos y otras especies. En este experimento, se evaluó la protección contra un desafío virulento del virus de la enfermedad infecciosa de la bolsa conferida por un virus adeno-asociado aviar expresando la proteína viral 2 (VP2) del virus de la enfermedad infecciosa de la bolsa (por sus siglas en Inglés rAAAV-VP2). Se inocularon aves libres de patógenos específicos con la recombinante rAAAV-VP2 o con una vacuna comercial intermedia del virus de la enfermedad infecciosa de la bolsa y luego fueron desafiados con la cepa Edgar. En todos los grupos vacunados se observaron anticuerpos específicos contra el virus de la enfermedad infecciosa de la bolsa. Los títulos de anticuerpos fueron mayores en el grupo vacunado con la vacuna comercial. La vacuna comercial a virus vivo indujo una protección adecuada contra morbilidad y mortalidad, sin embargo, tan temprano como el día 14 y hasta el día 28 cuando se desafiaron las aves, se observó depresión linfoide y atrofia folicular inicial asociada con la replicación viral activa. No se observó daño en el tejido de la bolsa debido a la vacuna recombinante rAAAV-VP2. Ocho de diez aves vacunadas con la recombinante sobrevivieron al desafío y no mostraron signos clínicos. El índice de peso bolsa:peso corporal y el registro de lesiones de la bolsa en el grupo vacunado con la recombinante rAAAV-VP2 indicaron protección. En consecuencia, la expresión transgénica de la proteína VP2 generó inmunidad protectora contra el desafío experimental en 80% de las aves, sin comprometer la bolsa de Fabricio. La utilización de viriones recombinantes del virus adeno-asociado aviar para transferencia genética es un nuevo enfoque para la vacunación en avicultura.

Key words: avian adeno-associated virus, vaccination, protection, infectious bursa disease

Abbreviations: AAV = adeno-associated virus; AAAV = avian adeno-associated virus; ELISA = enzyme-linked immunosorbent assay; HEK = human embryo kidney (cells); IBDV = infectious bursal disease virus; IgG = immunoglobulin G; IM = intramuscular; ORF = open reading frames; SNK = Student-Newman, Keuls test; rAAAV-VP2 = recombinant avian adeno-associated virus expressing the VP2 protein of IBDV; SPF = specific pathogen free

Infectious bursal disease virus (IBDV) is the etiological agent of the globally distributed infectious bursal disease, also known as Gumboro disease. The IBDV destroys the B lymphocyte precursors found within the bursa of Fabricius, inducing bursal atrophy, mortality, and immunosuppression in unprotected flocks (3). Very virulent IBDV strains emerged in Europe in the late 1980s; they can cause up to 60% mortality and are now considered a threat in several countries worldwide (1,7). The IBDV belongs to the family *Birnaviridae* (genus *Avibirnavirus*), which includes viruses with bisegmented dsRNA genomes (13). The IBDV genome is divided into segments A and B: segment A has two overlapping open reading frames (ORF); the first ORF of segment A encodes the non-structural protein VP5, probably involved in virus release and viral pathogenesis (9). The other portion of segment A encodes a precursor polyprotein in a large ORF, the product of which is cleaved by autoproteolysis to yield mature VP2 that forms the outer capsids of the virus. The VP2 is the major host-protective antigen of IBDV; it contains at least three independent epitopes responsible for the induction of neutralizing antibody (4). Segment A also encodes

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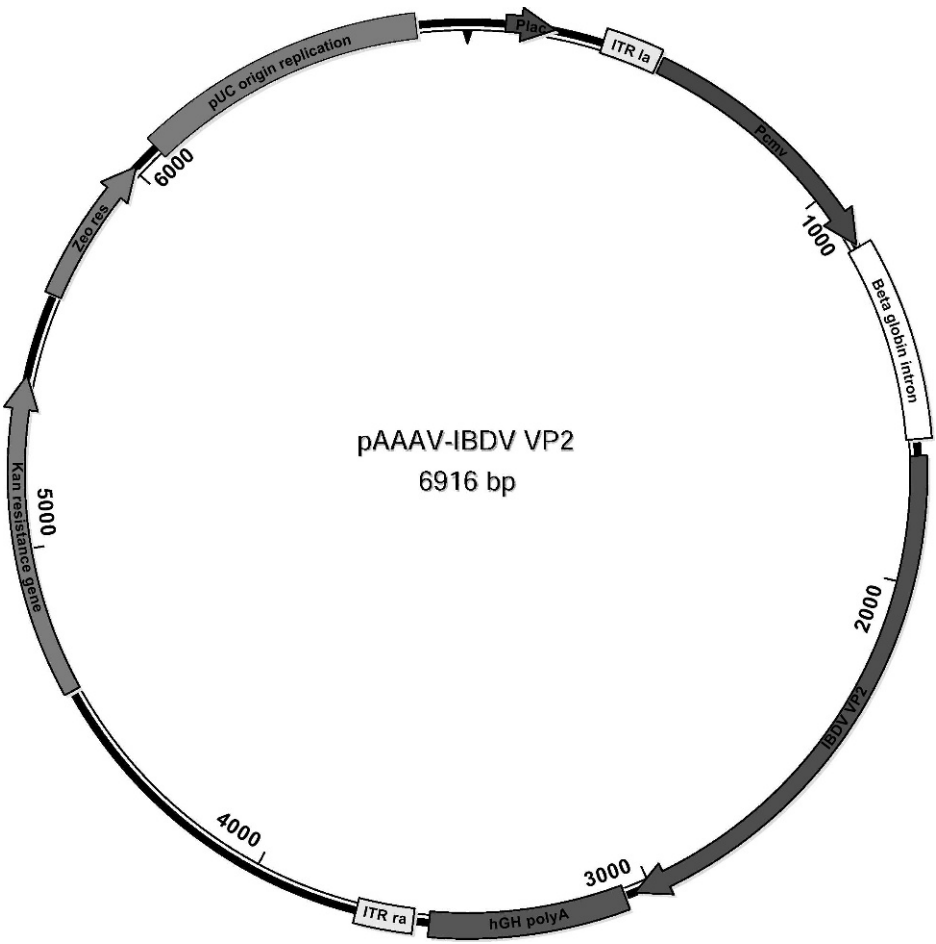

Fig. 1. Plasmid pAAAV IBDV-VP2, containing the Edgar strain VP2 gene under the influence of the late cytomegalovirus (CMV) promoter and flanked by the inverted terminal repeats (ITR) of the DA-1 strain of AAAV.

for the VP4 (protease) and the VP3 (inner capsid). Genome segment B encodes the virus polymerase VP1 (11).

Control of IBDV is currently attempted using live and killed vaccines for dams and offspring (3). Immunization of chickens against IBDV, with viral vectors expressing the VP2, has been previously reported and includes the use of avian herpesvirus (22), fowlpox virus (2), Newcastle disease virus (8), and fowl adenovirus (21) as expression systems. The advantages of the recombinant technology include safety (no reversion to pathogenicity of vaccine virus), that the vaccine can be tailor-made to match field virus phenotype, increasing its efficacy; and that a vaccine based on VP2 alone should allow monitoring of the field situation by the discrimination between antibody induced by vaccine (anti-VP2 only) and that induced by infection (anti-VP2 and VP3) (10,20).

The adeno-associated virus (AAV) is a replication-defective virus member of the family *Parvoviridae* that has been successfully used for gene delivery in humans and other species (23). The avian adeno-associated virus (AAAV) has been successfully used for transgenic expression of a reporter gene in chicken embryo tissues and cells (5,6). Recent work demonstrated the feasibility of generating recombinant AAAV virions expressing the immunogenic VP2 peptides of the Edgar strain of IBDV (rAAAV-VP2); it was also demonstrated that inoculation of SPF birds with these recombinant virions generates serological evidence of VP2 expression *in vivo* (14). The aim of this work was to evaluate the protection against a virulent IBDV challenge in SPF chickens vaccinated with rAAAV-VP2 virions and to compare this with commercial intermediate classic IBDV vaccination.

MATERIALS AND METHODS

Viruses. The intermediate classic vaccine ViBursa CE (Lohmann Animal Health. Winslow, Maine, U.S.A.) was used as recommended by the manufacturers. The Edgar strain used for the challenge was obtained from the Poultry Diagnostic and Research Center (Athens, GA, U.S.A.) stock and passed in 3-week-old SPF chickens' bursas to obtain a titer of 10^5 chicken infectious dose 50 per ml (CID_{50}/ml).

The AAAV-VP2 virions expressing the immunogenic VP2 peptides of the Edgar strain of IBDV were generated as previously explained (14). Briefly, rAAAV virions were generated by simultaneous transfection of human embryo kidney cells (HEK 293) monolayers with three plasmid constructs: 1) a plasmid designated p3.6 ITR-MCS-VP2, containing the Edgar strain VP2 gene under the influence of the late cytomegalovirus (CMV) promoter and flanked by the inverted terminal repeats (ITR) of the DA-1 strain of AAAV (Fig. 1); 2) a second plasmid expressing the Rep and Cap coding regions of AAAV designated pRC-CMV (6); and 3) a commercial pHelper plasmid coding for the E2, E4, and VR-RNA genes derived from the human adenovirus type 5 (Stratagene, La Jolla, CA, U.S.A.). The titer was determined by dot blot and reported as 1×10^{10} virus molecules/ml.

Experimental design. Ninety-six, 7-day-old SPF chickens were divided in six groups of 16 birds and placed in level two biosecurity units, where adequate husbandry was provided. Two groups were vaccinated by the intramuscular (IM) route, with 0.1 ml of the rAAAV-VP2 containing 10^9 virus molecules (groups 1 and 4). Another two groups (2 and 5) were vaccinated by eye drop with a commercial classic intermediate vaccine 10^5 CID_{50}/ml, and the last two groups (3 and 6) remained as nonvaccinated controls. At 14, 21, 28, and 35 days of age, eight birds from each group were randomly selected and tested for the

presence of anti-IBDV immunoglobulin G (IgG) using a commercial ELISA test (IDEXX Laboratories Inc., Westbrook, ME, U.S.A.), following manufacturer's protocols. On the same sampling days, two birds per group were sacrificed; body and bursa weights were individually recorded to obtain the bursa:body weight ratio (bursa weight:body weight × 100). Bursas were preserved in 10% buffered formalin for microscopic evaluation. At 28 days of age, the birds in groups 1, 2, and 3 (rAAAV-VP2, commercial, and nonvaccinated groups, respectively) were challenged by eye drop with 0.1 ml of virulent Edgar strain (10^4 CID_{50}/ml). The remaining birds (groups 4, 5, and 6) were not challenged and were used as treatment-specific nonchallenged controls. All birds were observed twice a day for seven days after challenge in order to record IBDV clinical signs and mortality.

Histopathology. The extent of bursal histologic damage was graded on a scale from 1 to 4, as previously described (19). Briefly, 1 = normal to 10% follicular atrophy; 2 = focal, mild, scattered cell depletion of up to 10%–30% follicular atrophy; 3 = multifocal follicular atrophy of 30%–70%; 4 = diffuse atrophy of >70% of the follicles or any evidence of acute necrosis.

Statistical analysis. All statistical analysis was performed using the Sigma Stat 3.0 software (SPSS, Chicago, IL, U.S.A.). Dunn's method and the Student-Newman, Keuls (SNK) test were performed at $P \leq 0.05$.

RESULTS AND DISCUSSION

Previous reports indicated that recombinant AAAVs induce the expression of a reporter gene in tissues derived from the rAAAV inoculated embryos (6), and that inoculation of SPF birds with a rAAAV vector expressing the VP2 of IBDV (rAAAV-VP2) stimulated a systemic humoral immune response measured as IBDV-specific ELISA titers (14). However, the ability of the immunity induced by rAAAV-VP2 to protect birds against infectious bursal disease remained to be clarified. In the present study, we therefore evaluated the level of protection conferred by rAAAV-VP2 vaccination in SPF chickens against a virulent challenge with the Edgar strain of IBDV. Morbidity, measured as the presence of IBDV clinical signs, mortality, serological response, bursa:body weight ratio, and the bursal lesion scores, was used as the criterion to assess protection; the results are summarized in Table 1.

Antibodies were detected by the IBDV-specific ELISA test as early as day 14 in all vaccinated birds. Statistically significant differences were observed at 14, 21, and 28 days of age between the vaccinated groups and the nonvaccinated controls. The serologic evidence of host recognition of the transgenic protein expression corroborated the suitability of the system for expression of poultry viruses' immunogenic proteins (6,14). When compared with the rAAAV-VP2 vaccinated birds, the antibody levels induced by the commercial vaccine were higher at all sampling points. These disparities can be explained by host-dependant differences in the immune mechanisms involved in the response to live IBDV replication (commercial vaccine) and the replication defective parvovirus used in the vector system (21).

Different levels of protection were observed within the challenged groups. In the nonvaccinated-challenged control group, 100% of the birds showed clinical signs; ruffled feathers, sick birds, and severe prostration were observed as early as day two after challenge. Furthermore, 40% of the birds died within the observation period. The macroscopic lesions included edema and hemorrhagic bursas observed three days after challenge strain; similar lesions have been reported when SPF birds are challenged with the Edgar strain (19). These kinds of lesions are also commonly observed with the highly pathogenic strains distributed in Europe, Asia, and Latin America (1,7).

In the rAAAV-VP2 vaccinated-challenged group, two-out-of-ten birds showed IBDV clinical signs and died, and eight birds remained healthy during the duration of the experiment, despite the challenge. Due to the replication-defective nature of the vector used, proper delivery to the host cells is required to ensure target protein expression; inadequate IM inoculation or poor individual response to vaccination of these two SPF birds may account for the lack of efficacy. Other possible explanations include: 1) a previously reported tendency of recombinant adeno-associated virus particles to agglutinate spontaneously; the aggregation of recombinant AAAV seems to be directly associated with variability in levels of empty capsids and DNA; or 2) protein impurities in the vector preparations, leading to reduced yield and less efficient gene transfer (23). In this case, due to the agglutination-induced gene transfer decrease, the threshold of protein expression required to mount an effective immune response may not have been reached in the two birds that were not protected; further experiments are required to clarify this point.

After challenge, the bursa:body weight ratios of the birds surviving in the rAAAV-VP2 group were significantly ($P < 0.05$) higher and the bursal lesion scores lower than those of the commercial vaccinated and unvaccinated control groups, indicating adequate protection. None of the birds inoculated with the commercial vaccine showed clinical IBDV or died after challenge; these results are in agreement with previous reports in which the efficacy of Lukert strain-derived vaccines against IBDV virulent challenge has been demonstrated (10,15).

The histopathology of the bursas of Fabricius, seven days after challenge, is shown in Fig. 2. Nonchallenged groups are shown for comparison. The nonvaccinated-challenged group showed severe lymphoid depletion of the follicles, an increased amount of stroma between follicles, and severe follicular atrophy. The commercial vaccine group showed moderate lymphoid depletion in many follicles and some signs of lymphoid repopulation. The rAAAV-VP2 challenged group showed mild lymphoid depletion of the follicles, indicating no challenge-derived tissue damage. Similar levels of protection have been previously reported for other recombinant vaccines expressing the immunogenic VP2 protein (2,8,20,21).

The three groups that remained unchallenged (groups 4, 5, 6) showed no IBDV clinical signs during the entire experiment. When compared with their homologous challenged groups at day 35, the bursa:body weight ratios were higher and the bursal lesion scores lower, demonstrating the effect of the virus used in the challenge and validating the results obtained in the challenged groups. The commercial vaccine group showed moderate to severe lymphoid depletion of the follicles. No lesions were observed in the bursas from the rAAAV-VP2 vaccinated groups, in accord with what has been previously reported for other IBDV recombinant vaccines (2).

Live vaccines against IBDV have been proved to generate very good protection against the disease. Nevertheless, due to the worldwide emergence of very virulent IBDV, the industry has resorted to the use of less attenuated vaccines that can compromise the bursal integrity of the young bird and may affect its ability to respond against other pathogens or standard vaccination programs (12,18). The results indicate that, although transgenic VP2 expression induces measurable immune responses, the recombinant viruses do not target bursal B lymphocytes, representing the most important competitive advantage over live vaccines that are prepared using virus strains with increased pathogenicity (16,18).

The bursal tissue integrity observed after rAAAV-VP2 vaccination correlates with the expectation of an adequate response to

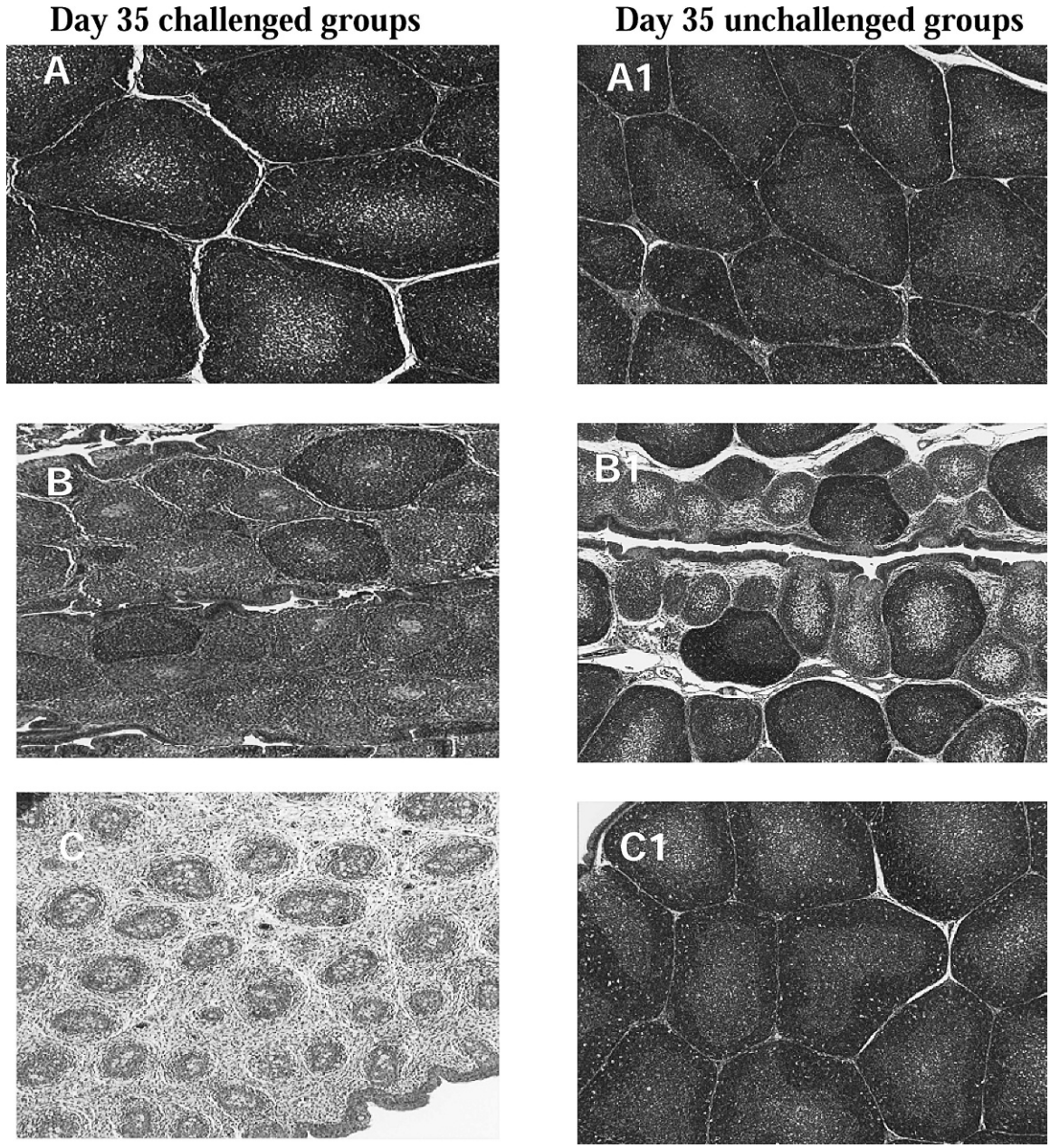

Fig. 2. Histopathology of the bursa of Fabricius at day 35. Challenged groups: rAAAVP2/challenged (A) = mild lymphoid depletion of the follicles. Commercial vaccine/challenged (B) = moderate lymphoid depletion of the follicles. Nonvaccinated/challenged (C) = severe lymphoid depletion of follicles, increased amounts of stroma between follicles and severe follicular atrophy. Nonchallenged groups: rAAAVP2 (A1) = no lymphoid depletion of the follicles. Commercial vaccine (B1) = lymphoid depletion in many follicles and lymphoid repopulation of some follicles. Nonvaccinated/nonchallenged (C1) = normal bursal histology. (H&E. 100×)

vaccination programs or to field challenges after initial IBDV vaccination (17). Therefore, early and frequent vaccination with intermediate or intermediate-plus vaccines can induce high levels of IBDV neutralizing antibodies, but can also deploy the bursal B cell population, thus generating problems with immune responses against other pathogens and a failure of vaccination programs (10,17).

This pilot study only evaluated the protection after parenteral application and against classical IBDV viruses; nevertheless, initial investigations revealed a humoral immune response after *in ovo* application of rAAAV-VP2 particles (14). Also, the possibility of generating a broad spectrum vaccine by including both classical and variant VP2 proteins is sound. A tandem copy of the genes of interest, with an internal ribosomal entry site, will generate a bi-cistronic mRNA to express both classical and variant VP2 proteins. Although the 80% protection obtained in this trial is close to the 90% protection expected in an efficacy test for IBDV vaccines, the number of birds used in this proof-of-concept study was limited, and further trials using a larger number of birds are required. Overall, this experiment demonstrated that the rAAAV-based transgenic expression of the IBDV VP2 protein in SPF birds induces protective immunity against IBDV virulent challenge, and that vaccination with the rAAAV-VP2 virions did not affect bursal lymphoid tissue, demonstrating its potential for use in young chickens.

Table 1. Comparison of the results from the rAAAV-VP2 and commercial vaccine vaccination/challenge trial.

Treatment[A]	Day 14 of age			Day 21 of age			Day 28 of age			Day 35 of age			Clinical protection against challenge	
	ELISA titers[B]	Bw/Bw index[C]	Bursa score	ELISA titers	Bw/Bw index	Bursa score	ELISA titers	Bw/Bw index	Bursa score	ELISA titers	Bw/Bw index	Bursa score	Morbidity %	Mortality %
rAAAV-VP2 challenged at day 28	775[a]	0.53[a]	–	920[a]	0.60[a]	1.0	1100[b]	0.85[a]	1.5	3250[b]	0.54[b]	2.5	20	20
Commercial challenged at day 28	685[a]	0.30[b]	–	1049[a]	0.28[b]	3.0	2805[a]	0.19[b]	3.0	4581[a]	0.19[c]	3.0	0	0
Control challenged at day 28	0[b]	0.55[a]	–	0[b]	0.73[a]	1.0	0[c]	0.63[a]	1.5	2020[a]	0.20[c]	4.0	100	40
rAAAV-VP2 unchallenged	599[a]	0.54[a]	–	796[a]	0.55[a]	1.0	980[b]	0.63[a]	2.0	1065[a]	0.64[a]	2.0	–	–
Commercial unchallenged	923[a]	0.27[b]	–	1043[a]	0.33[b]	3.0	2977[a]	0.26[b]	3.0	3100[a]	0.24[c]	2.5	–	–
Control unchallenged	0[b]	0.53[a]	–	0[b]	0.59[a]	1.0	0[c]	0.77[a]	1.5	0[a]	0.66[a]	1.5	–	–

[A]Means with the same letter within column are not significantly different by the SNK test ($P < 0.05$).
[B]ELISA antibodies expressed as geometric mean (GMT) titers.
[C]Bursa:body weight ratios calculated using the following formula: Bursa weight:body weight × 100.

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